

*INTERIM
7/11/87
80269***Year 1 Annual Report**

Grant #: IRP 95-101

Title: Quantitation of Bone Growth Rate Variability in Rats Exposed to Micro- (near zero G) and Macrogravity (2G)

Investigators:

Timothy G. Bromage (Hunter College)
Stephen B. Doty (Hospital for Special Surgery)
Igor Smolyar (Hunter College)
Emily Holton (NASA)

INTRODUCTION**Aims**

Our stated primary objective is to quantify the growth rate variability of rat lamellar bone exposed to micro- (near zero G: Cosmos 1887 & 2044; SLS-1 & SLS-2) and macrogravity (2G). The primary significance of the proposed work is that an elegant method will be established that unequivocally characterizes the morphological consequences of gravitational factors on developing bone. The integrity of this objective depends upon our successful preparation of thin sections suitable for imaging individual bone lamellae, and our imaging and quantitation of growth rate variability in populations of lamellae from individual bone samples.

Year 1: Expectations

We explained that we would set up and gain familiarity with the specimen preparation protocol and image analysis system in the Hard Tissue Research Unit, Hunter College. Student assistants would be trained in routine operations. Specimen preparation and analysis would be undertaken on rat long bone "practice" specimens in order to affirm our proficiency (specimen preparation would thence become routine for the two year project).

In our proposal we described a systematic investigation of labeled SLS-1 and SLS-2 bone in which the lamella formation rate would be determined. Quantitative analyses of lamellae bordering vital labels would be undertaken in order to characterize any effect the fluorochromes may have had on bone formation rates. These studies were a prelude to the quantitative analyses of lamellar growth rate variability in Year 2.

Year 1: Actual

We have accomplished our research objective. While we expected that much of our initial effort would be spent on specimen preparation, however, this was not to be. NASA hoped that despite restructuring our 2 year budget into 3 years, vendors would be motivated to provide the necessary specimen preparation equipment anyway, given partial payment and the promise of subsequent payments during Year 2 and Year 3. Our equipment vendor refused to supply us (we were told of great unease in the private sector regarding Congressional threats to cut allocations to NASA - and to science in general - in the Fall of 1995, and nothing we said seemed to mollify them). We supplied a commercial laboratory with a test sample for preparation, but the quality of their thin section was inferior to our needs. We have not, therefore, begun to prepare sections according to our stated requirements.

Sections were prepared that could satisfy the simpler objectives of Year 1 and, for this, we have determined the lamella formation rate for the SLS experimental rat (see Results, below). This has also allowed us to make some preliminary observations about the potential effect that fluorochromes have on bone formation rates.

Given the delay in refined specimen preparation, we worked very hard in other ways to prepare for the quantitative aspect of our research to begin in Year 2. First, we have configured a series of image processing steps that provide a binary "working" image. This was possible given the willingness of our image analysis provider to supply us with some of the equipment necessary. Second, in our proposal we described a novel method for examining bone formation rates. We are now capable of executing this method by means of a Windows®-based program that we have developed for automating the measurement taking and for configuring a pseudo 3-D chart illustrating bone growth rate variability with precision to 24 hours (see Figure: Quantification of the rat bone growth rate).

MATERIALS AND METHODS

Animals

Specimens from the following experiments were investigated:

Experiment: SLS-1

Experimental groups: 19 RAHF Flight and Vivarium Control

Mission Length (ML): 9 day flight (ML=9)

Species: male Harlan (Sprague/Dawley)

Bone Element: labeled humerus

Experiment: SLS-2

Experimental groups: 20 Flight and Vivarium Control

Mission Length (ML): 14 day flight (ML=14)

Species: male Harlan (Sprague/Dawley)

Bone Elements: labeled humerus

Each experimental group had a proscribed vital labeling regime of Calcein and Demeclocycline prior to and around Launch, and at Recovery.

Thin sections cut from plastic embedded samples were very carefully ground on 1200 grit paper to 80-100 microns and cleaned with Tergazyme (Alconox, NJ) enzyme detergent at 50° C for 24 hours. Sections were mounted in water and imaged by polarizing and fluorescence microscopy.

For this work we focus on a little studied, but fundamental, microanatomical feature of bone - the bone lamella. Bone lamellae represent successive forming fronts during the development in width of a long bone. Measurements of lamellar widths are direct measures of growth rates. These measurements are unique to individuals and do not depend upon interindividual variability. A single bone lamella is defined by us as a linear (in the cross sectional sense) breadth of mineralized matrix comprising one collagen fiber domain with fibers running predominantly in one direction, and another domain with fibers running at some significant angle from the direction of the first domain. Thus we use polarized light microscopy to identify, in our illustrative example (see Figure: SLS-1-57 polarized light image), a bright domain comprising fibers running parallel with the plane of section, and a dark domain comprising fibers running more longitudinal with the plane of section. (There are notable exceptions to this definition which would, then, require other forms of polarizing microscopy such as circularly polarized light, to achieve the necessary contrast).

Instruments

Images of endosteal lamellae were obtained with a Leica DMRX\E light microscope fitted with linear polarizing filters and a 100 Watt fluorescence system. Images were retrieved by a Kodak CCD camera and transported to the Leica Quantimet 600, a framestore-based image analysis system. An analytical program was written which standardized gray level image enhancement and filtering procedures. The resulting image was then passed to the quantification program for analysis (below).

Quantification Software

We developed software (C compiler for DOS environment) for processing lamellar bone. The input for this software is binary image of a bone. The output is a 3-D chart (Appendix) which describes the variability of the bone growth rate.

ANALYSES

Lamella Formation Rate

The results of our microscopy and analyses of labeled SLS bone are illustrated by example in the Figures of SLS-1-57 (polarized light image, fluorescence image, and processed image). The most important and interesting result is that endosteal lamellae of the humerus (and we expect in many other locations) of the juvenile rat accrue at the rate of 1 lamella per day. In other words, the lamella formation rate follows a circadian rhythm. We, in fact, find this rather astonishing. This is,

additionally, the first calculation of a lamellar formation rate in the mineralized tissue sciences. The polarized light and fluorescence images are annotated with information learned from our analysis and summarized on the final processed image. It became clear that we could calibrate the bone on the basis of the Calcein labels which are reasonably sharp and identifiable to individual lamellae.

We have taken note, however, that the vital labels employed bind to the mineral phase and, therefore, fluorescence will be indicated at some minor depth behind the organic - i.e. non-mineralized - bone forming front. The precise number of days between Calcein labels equals the number of lamella between them, thus one lamella equals one day. Knowing, in this case of SLS-1, that nine days followed Recovery and the last Calcein label, we are able to calibrate the remaining events in the life history of the individual. We are also able to reason that there exists a circa one day mineralization lag (i.e. the osteoblastic layer is one day - or one lamellae width - beyond the mineralizing front and lining atop the matrix previously formed by these cells). Note, for instance, in the fluorescence image, that the administration date of 6/14-91 Calcein is shown immediately above its fluorescence (faint blue in this false color image) where immature mineral is taking up Calcein in this region. Likewise, 6/22 mineralization is still underway on the 6/23 euthanizing date, and no 6/22 to 6/23 lamella is visible.

Effect of Vital Labels on Bone Formation

Our preliminary results suggest that the administration of vital labels Calcein and Demeclocycline is very slightly hindering osteoblastic activity. We provide here a pseudo 3-D chart of the bone growth rate variability (see Figure: Quantification of the rat bone growth rate) indicating growth rate in the Z direction, time in the X direction, and sampling error in the Y direction. The chart illustrates decreases in the widths between lamellae at times of administrations of labels in SLS-1-57. Each Calcein label is associated with a marked short-term downward trend in the Z direction followed by a sharp recovery in the growth rate. Each Demeclocycline label is also characterized by a drop in bone formation rate but not to the same extent as demonstrated in response to the administration of Calcein. Recovery in the growth rate is rapid in the case of the first Demeclocycline label but it is less so in the second. The pseudo 3-D chart of the control rat may be interpreted similarly, though, to date, we have investigated little of this sample in respect to this, our Year 2 objective. In the case of SLS-1-72 there are also decreases in the bone formation rate at bone Calcein labeling intervals (1st and 4th labels) and two downward trends are evident at the Demeclocycline labeling intervals (2nd and 3rd labels). Specimen preparation procedures were not optimal and this image is noisy in respect to prepared flight specimens. In order to confirm these findings we must conduct a more extensive search of images prepared according to our proposed specimen preparation protocol.

Effect of Space Flight on Bone Formation

It has not gone unnoticed that in our preliminary study of the effect of vital labels on bone formation, illustrated by our pseudo 3-D chart, we have also retrieved some initial observations on the effect of space flight on bone formation with 24 hour resolution. The second Demeclocycline label was administered two days before Launch. We would expect under conditions of 1G that the growth rate would recover as it did after the first Demeclocycline label. This recovery, however, was hindered initially, we think, by the stress of Launch and the first day subject to microgravity. Thereafter the

growth rate recovered only slightly, after approximately five days, to a relative growth rate still lower than exhibited by normal pre- and postflight bone. During the second half of the Mission the growth rate adopted a slow downward trend. The circum-Recovery period is characterized by a sharp drop in bone formation following Calcein administration (see above) and then a continuous rise in the bone formation rate until euthanized.

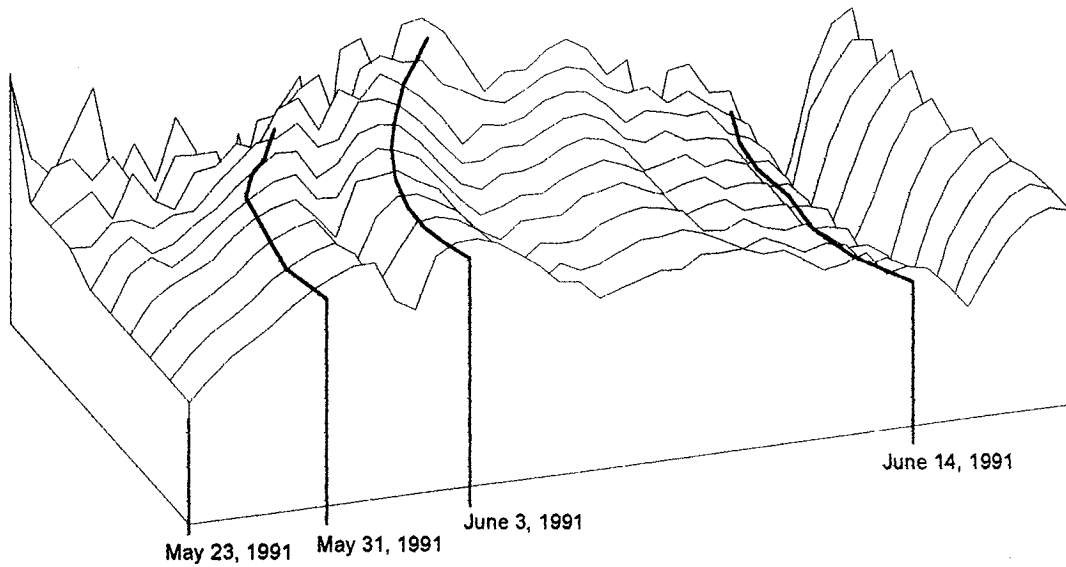
YEAR 2

Year 1 work has enabled us to proceed freely with our anticipated Year 2 objectives which are to undertake quantitative analyses of lamellar growth rate variability of labeled and unlabeled SLS and Cosmos mission bones and their controls. Although our specimen preparation equipment was on hold, the vendor now feels that, as the Year 2 budget is so near, we will now be accommodated.

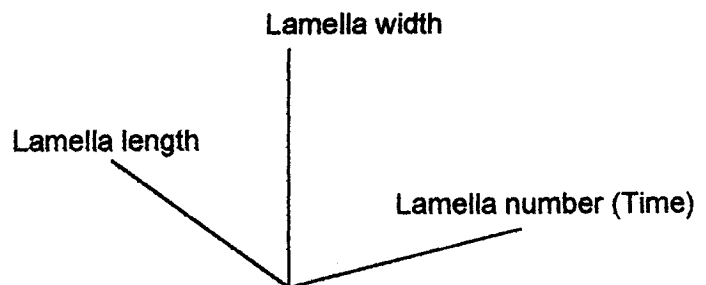
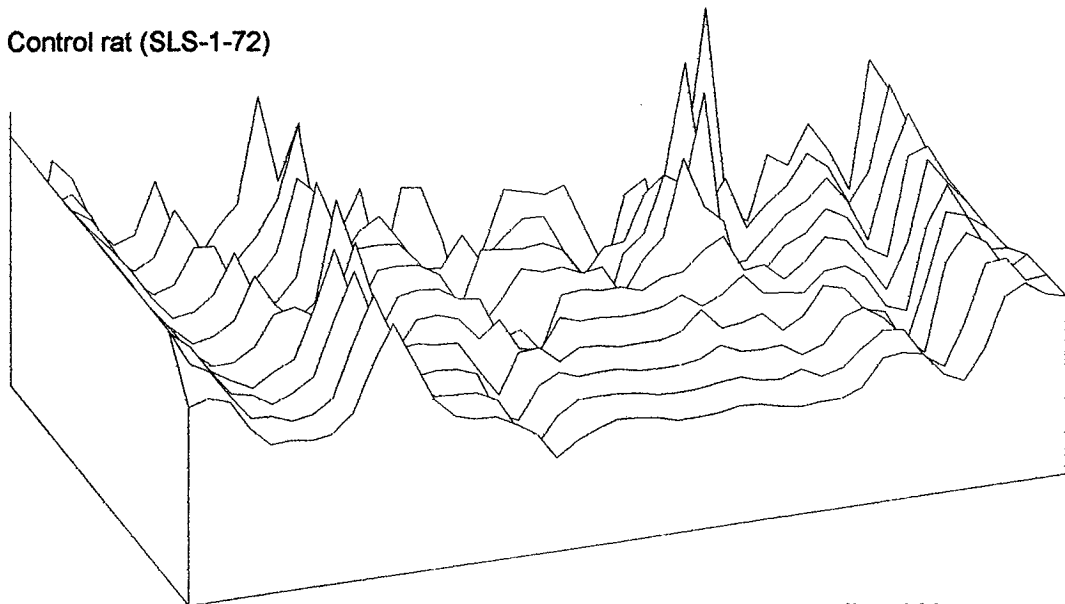
Backscattered electron microscopy of bone specimens will also be undertaken in order to identify, with very high precision (i.e. 24 hours), differences in bone mineral density that are dependent upon gravitational factors.

QUANTIFICATION OF THE RAT BONE GROWTH RATE

Flight rat (SLS-1-57)



Control rat (SLS-1-72)



SLS-1-57

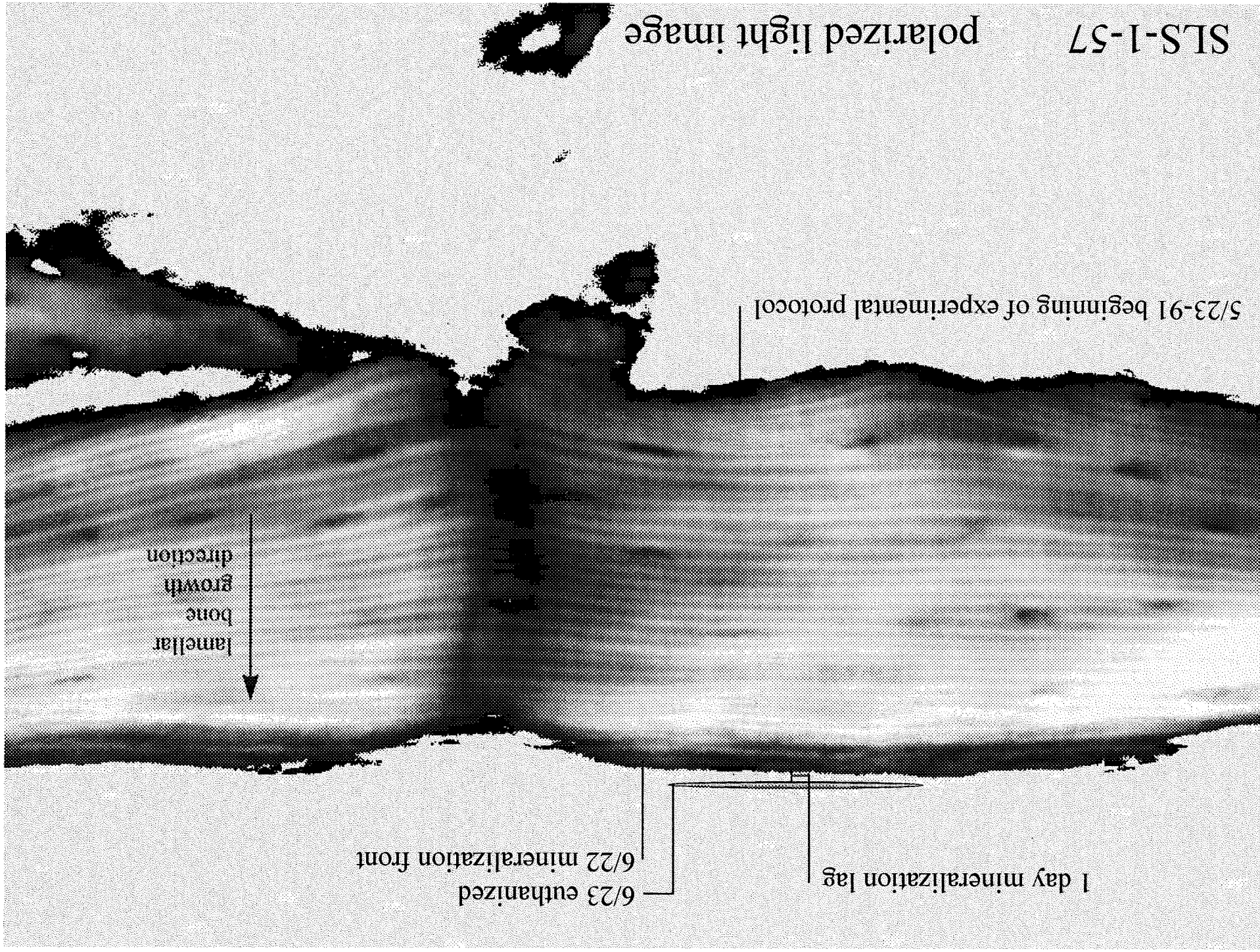
polarized light image

5/23-91 beginning of experimental protocol

lamellar
bone
growth
direction

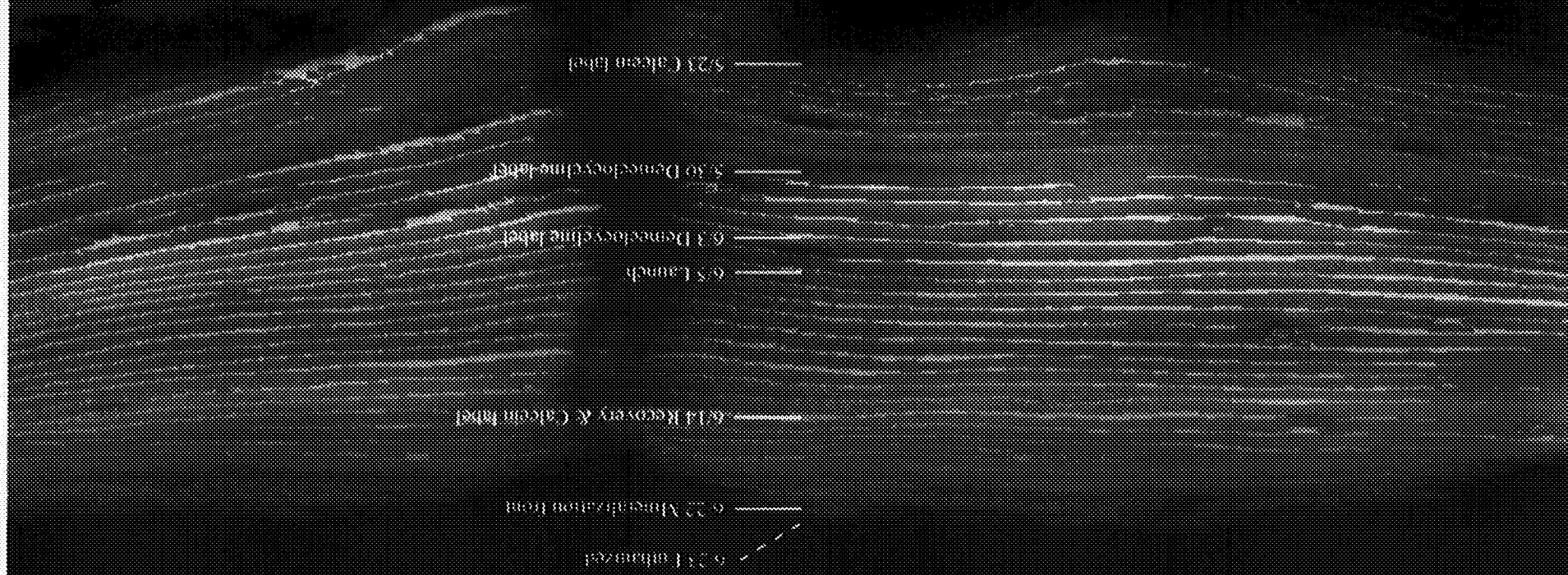
6/23 euthanized
6/22 mineralization front

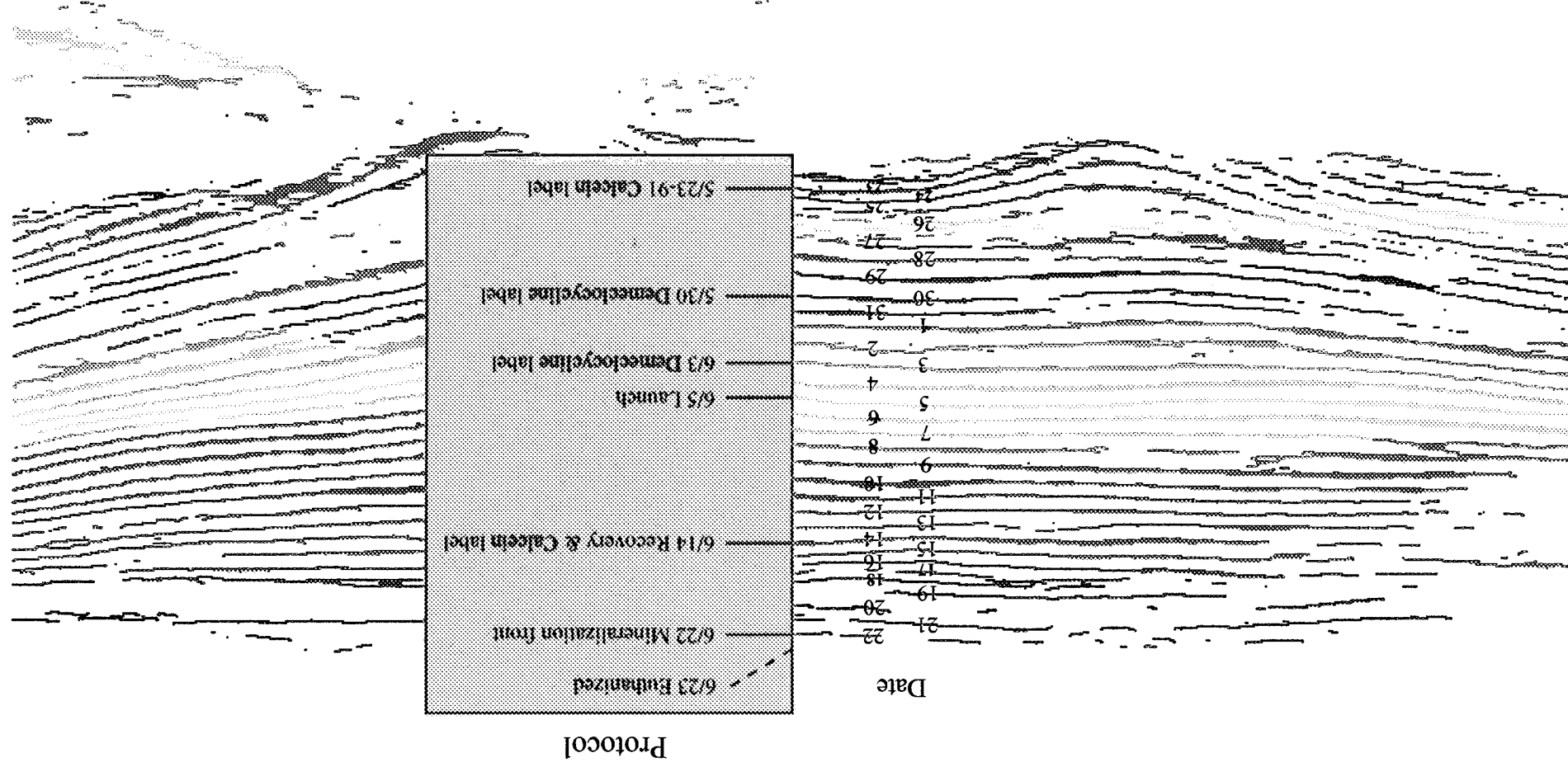
1 day mineralization lag



SLS-1-57

fluorescence image





processed image

SLS-1-57